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## Japanese encephalitis virus immunoglobulin M antibodies in porcine sera

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### SUMMARY

A solid-phase enzyme-linked immunosorbent assay (ELISA) was developed for detection of porcine immunoglobulin (Ig)M antibodies to Japanese encephalitis virus (JEV). Antibodies in sera were captured onto the solid phase of Microtiter plates sensitized with mouse monoclonal antibodies to porcine  $\mu$  heavy chain. Virus antigen binding to the lawn of IgM was quantitated by subsequent binding of peroxidase-labeled human hyperimmune anti-JEV IgG, which in the final step, catalyzed a substrate color change.

In sucrose density-gradient fractionated sera from recently infected pigs, the peak of ELISA JEV IgM activity corresponded to the peak of 18-S, 2-mercaptoethanol-sensitive hemagglutination-inhibiting (HAI) antibody activity. Within 2 to 3 days, JEV-infected sentinel pigs developed high JEV IgM activity; this activity decreased within 2 weeks.

Among specimens collected from 99 random swine at abattoirs in Thailand during a period of low JEV transmission, none of 25 JEV HAI-negative sera had JEV IgM activity, 7 of 74 JEV HAI-positive sera did have JEV IgM activity, and the remaining 67 sera had readily detectable JEV HAI antibodies, but lacked JEV IgM. The JEV IgM solid-phase ELISA was useful for rapidly diagnosing active or recent JEV infections in swine.

Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that causes epidemics of encephalitis in human beings throughout Asia<sup>1</sup>; in Thailand, swine are the major intermediate amplifying host.<sup>2</sup> Although infected adult swine typically have no clinical signs of illness, infected pregnant sows may abort or absorb fetuses.<sup>3</sup> Laboratory tests capable of identifying pigs with current or recent JEV infections are important in monitoring JEV transmission rates and in assessing the economic impact of JEV on swine reproduction. The 3 methods currently available

have major disadvantages: (i) virus isolation from blood requires highly specialized laboratory facilities and is time-consuming; (ii) demonstration of an increasing serum antibody titer requires that a 2nd blood sample be taken after 7 to 10 days, causing an intrinsic delay; and (iii) detection of JEV immunoglobulin (Ig)M antibodies by demonstration of a decrease in the serum hemagglutination inhibition (HAI) titer after treatment with 2-mercaptoethanol (2-ME) is insensitive.

Immunoglobulin M antibodies to JEV appear in the serum early after infection and then rapidly decrease.<sup>4,5</sup> The purposes of the present report were to develop a solid phase enzyme-linked immunosorbent assay (ELISA) for the detection of swine IgM antibodies to JEV and to determine JEV IgM activity in a single serum specimen required to establish a diagnosis of JEV.

### Materials and Methods

**HAI serologic studies**—Serologic studies were performed as described previously,<sup>6</sup> but were modified for a Microtiter system. All sera were extracted with acetone and absorbed with goose RBC before testing. Sucrose and acetone extracts of pooled suckling mouse brains were used as antigens. Passage 17 JEV Nakayama strain was used. Whole sera were tested at a dilution of 1:10 and at 2-fold dilutions thereafter. Specimens fractionated by sucrose density-gradient centrifugation were absorbed, but not extracted. The lowest dilution of gradient fractions tested was 1:4. Samples of gradient fractions were tested in the absence and presence of 0.1M 2-ME for 60 minutes.

**Sucrose density-gradient fractionation**—Sera (0.3 ml) were subjected to rate zonal ultracentrifugation<sup>7</sup> through 4.8 ml of continuous 10% to 40% sucrose gradients for 18 hours at 39,000 rpm. Twelve fractions, each approximately 0.4 ml, were obtained by puncture of the tube bottom.

**Monoclonal antibody to porcine  $\mu$  chain**—Murine ascitic fluid 5C9.C12.1, containing a  $\gamma$ -1/ $\kappa$  monoclonal antibody to porcine  $\mu$  chain,<sup>8</sup> was used.<sup>9</sup>

**Performance of the JEV IgM solid-phase ELISA**—The ELISA was performed in 5 steps.

**Sensitization**—All 96 wells of a disposable polystyrene U-bottom Microtiter plate<sup>10</sup> were sensitized with 100  $\mu$ l of mouse monoclonal antibody (5C9.C12.1) diluted 1:1,000 in 0.006M sodium carbonate buffer (pH 9.0) for 4 hours at room temperature. The

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<sup>8</sup> Dr. R.A. Van Deusen, National Veterinary Services Laboratories, Ames, Iowa.

<sup>9</sup> Linbro Scientific Co, Hamden, Conn.

plates were washed 3 times with 0.05% Tween-80<sup>c</sup> in phosphate-buffered saline solution (PBSS).

**Test sample**—The test sample serum (50  $\mu$ l), diluted to 1:100 in 0.5% bovine serum albumin<sup>d</sup> (BSA) in PBSS, was added to each well and was incubated for 2 hours at room temperature. Test specimens were not pretreated. Plates were again washed 3 times with PBSS-Tween 80.

**Antigen**—Sucrose- and acetone-extracted pooled suckling mouse brain JEV antigen (50  $\mu$ l containing 50 hemagglutinating units), diluted in a solution of 20% acetone-extracted normal human serum plus 80% PBSS-BSA, was added to each well and was incubated overnight at 4 C. The plates were then washed 5 times with PBSS-Tween 80.

**Label**—A 1:500 dilution of horseradish peroxidase (type VI<sup>d</sup>)-labeled human flavivirus hyperimmune IgG in 20% normal human serum in PBSS-BSA (25  $\mu$ l) was added to each well and was incubated for 2 hours at room temperature. Human IgG was used because this was the highest titered antisera available (HAI titer > 1:5,120). The IgG and horseradish peroxidase were coupled by the 2-step glutaraldehyde method.<sup>e</sup> Normal human serum in the diluent acted as a quenching agent to inhibit competitively nonspecific binding of labeled IgG. The plates were washed 7 times with PBSS-Tween 80.

**Substrate**—Orthophenylenediamine<sup>d</sup> substrate solution<sup>e</sup> (100  $\mu$ l) was added to each well and was incubated at room temperature for 30 minutes, at which time the reaction was stopped by the addition of 50  $\mu$ l of 4N sulfuric acid. The absorbance of each well at 492 nm was read in an automated plate reader.<sup>f</sup>

Multiple serum samples from 1 pig were always tested on the same immunoassay plate. When serial dilutions were performed, sera or gradient fractions were diluted 10-fold in PBSS-BSA. In some control ELISA, 50 hemagglutinating units of other prototype flavivirus suckling mouse brain antigens were used: Wesselsbron, Langat, Tembusu, and Dengue type 2, which are all native to Thailand.<sup>g</sup>

**Pig sera**—Two collections of sera were examined. One collection consisted of 96 serum samples drawn at 2- to 4-day intervals from ten 6- to 10-month-old sentinel pigs in Choomporn, Thailand. Eight to 11 serum samples were drawn from each pig between July 13 and Aug 5, 1983. As detected by conventional HAI serologic study, 7 of 10 sentinel pigs became infected with JEV. Japanese encephalitis virus and not some other flavivirus proved to be the infecting virus type by isolation and identification of the virus from sera collected and stored during the week before HAI antibodies became detectable (data not shown). The 2nd collection consisted of 1 serum sample drawn from each of ninety-nine 6- to 12-month-old live swine (random source) at abattoirs in the southern Thailand provinces of Choomporn, Surat Thani, and Prachuab Khiri Khan in May of 1983. These sera were tested twice by ELISA.

**Definition of JEV IgM units**—Specimens with no, low, and high JEV IgM activity were chosen as negative, weak-positive, and strong-positive control sera, respectively. These sera were tested on all subsequent plates to control plate-to-plate variations in test results. The absorbance values at 492 nm were approximately 0.05 for negative, 0.40 for weak-positive, and 2.00 for strong-positive control sera. The weak-positive control specimen was one that produced an absorbance value estimated to be near the cut-off value for defining JEV IgM-positive and -negative sera. Dimensionless JEV IgM activity units were defined as the ratio of the absorbance of the final color product obtained with the test specimen divided by the absorbance of the final color product obtained with the weak positive control serum (mean of 4 wells), multiplied by 100. Thus, by definition, any specimen yielding an absorbance equal to that of the weak positive-control serum had 100 U of JEV IgM activity.

<sup>a</sup> Sigma Chemical Co, St Louis, Mo.

<sup>b</sup> Titertek Multiskan M, Flow Laboratories, McLean Va.

## Results

**JEV IgM in sentinel pig sera**—None of 3 pigs that remained JEV HAI antibody-negative developed JEV IgM, whereas postinfection sera from all 7 infected pigs promptly (within 2 to 3 days after viremia) had strong JEV IgM reactivity. The intensity of the JEV IgM reactivity was maximal about 1 week later, then rapidly decreased to low activity within 2 to 3 weeks, whereas the HAI titers remained high (Fig 1).

**Density-gradient fractionation**—Preinfection, immediate postinfection (5 to 6 days), and late postinfection (2 weeks) sera from 2 sentinel pigs were analyzed by sucrose density-gradient fractionation. The largest peaks of 18-S, 2-ME-sensitive HAI antibodies were found, using immediate postinfection sera. The peak of ELISA JEV IgM activity cosedimented with the peak of 18-S, 2-ME-sensitive JEV HAI antibodies. An additional peak of immunoassay activity was not found to cosediment with a distinct 7-S, 2-ME-insensitive HAI peak (Fig 2).

**JEV IgM in abattoir swine sera**—Of the 99 sera collected at the abattoirs, 74 had JEV HAI antibody titers of > 1:10 (geometric mean titer = 1:80), indicating past infection with JEV or a related flavivirus. All 25 JEV HAI-

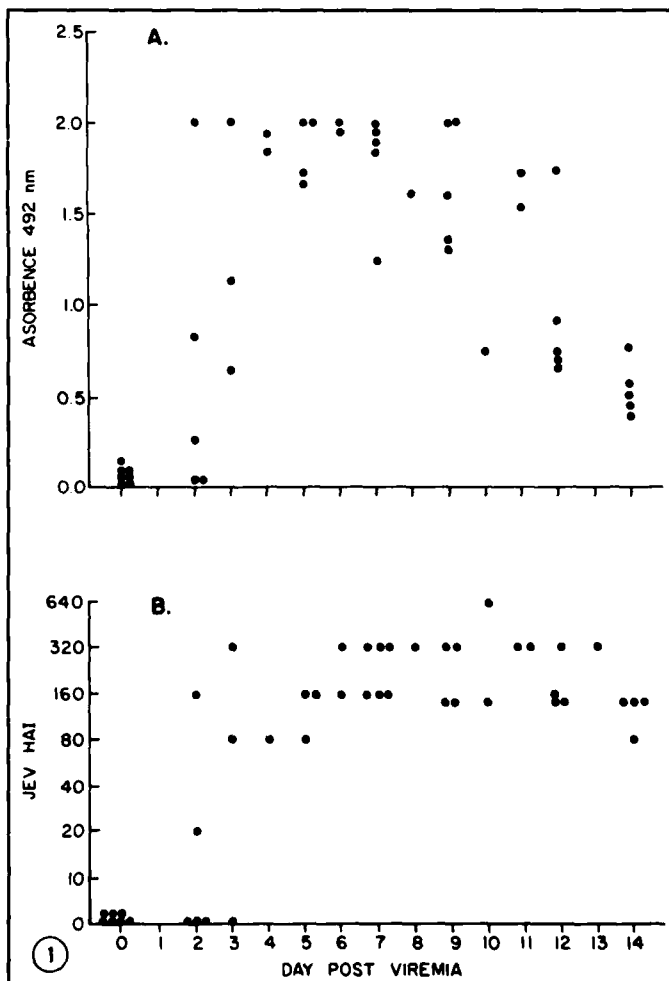


Fig 1—Time course of JEV antibodies in 7 infected sentinel pigs. Each point represents a single measurement. Absorbance in JEV IgM ELISA, using a 1:100 dilution of sera (A). The JEV HAI titer of sera (B).

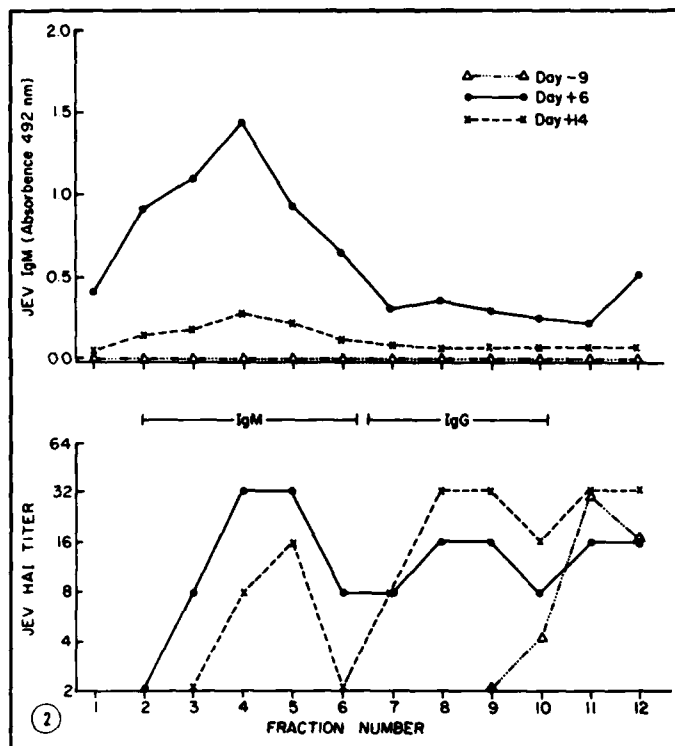


Fig 2—Sucrose density-gradient fractions of preinfection, immediate post-infection, and late postinfection sera tested for JEV HAI activity (bottom panel) and ELISA JEV IgM activity (top panel).

negative sera contained  $< 100$  U of JEV IgM. Of 74 JEV HAI-positive sera, 7 contained  $> 100$  U of JEV IgM; in 2 sera, the activity was strong ( $> 500$  U, absorbance values  $> 2.00$ ), whereas in 5 sera, the JEV IgM activity was only slightly increased ( $> 100$  but  $< 250$  U, absorbance values 0.3 to 0.8). The modal JEV IgM activity was 11 to 20 U for HAI-positive and -negative sera. The mean JEV IgM units ( $\pm 1$  SD) of the 25 HAI-negative specimens was  $20 \pm 19$ ; the mean of the 67 HAI-positive, JEV IgM-negative ( $< 100$  U) specimens was  $26 \pm 19$ . Among HAI-positive specimens, sera with high JEV HAI antibody titers ( $\geq 1:160$ ) were more likely to contain JEV IgM activity than were sera with low JEV HAI titers (6/30 vs 1/44,  $P = 0.015$  by Fishers exact test, Fig 3). Both of the strongly reactive, and 4 of the 5 weakly reactive abattoir swine sera contained 18-S, 2-ME-sensitive, JEV HAI antibodies when fractionated through sucrose-density gradients.

**Specificity of reagents**—All 7 JEV IgM-containing sera collected at the abattoirs and the negative, weak-positive, and strong-positive control sera were diluted 1:100 and were tested in 3 control ELISA. In each control ELISA, 1 reagent was varied: (i) nonimmune mouse ascitic fluid was substituted for ascitic fluid 5C9.C12.1, (ii) normal suckling mouse brain antigen was substituted for JEV-infected mouse brain antigen, or (iii) human hyperimmune anti-hepatitis B core horseradish peroxidase-labeled IgG was substituted for the human hyperimmune anti-flavivirus peroxidase-labeled IgG. In all cases, the absorbance values were  $< 0.2$ . Seven strongly reactive sentinel pig sera and all 7 positive abattoir sera were retested, using Wesselsbron, Langat, Tembusu, or Dengue 2 antigens in place of JEV antigen. Resultant absorb-

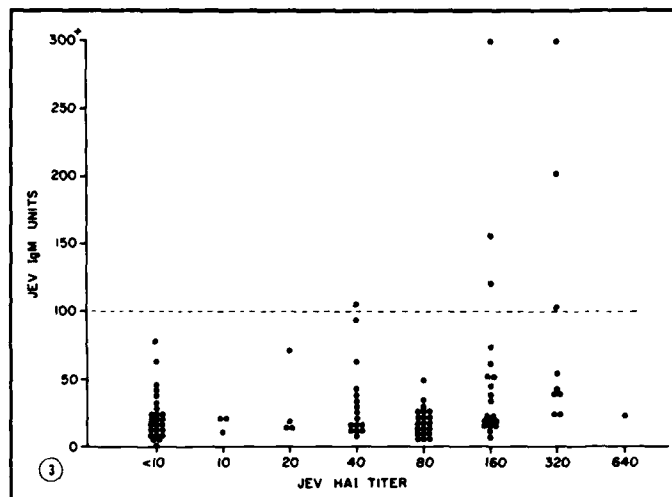


Fig 3—Porcine sera collected at abattoirs: Relationship of JEV HAI titer and ELISA JEV IgM activity (units).

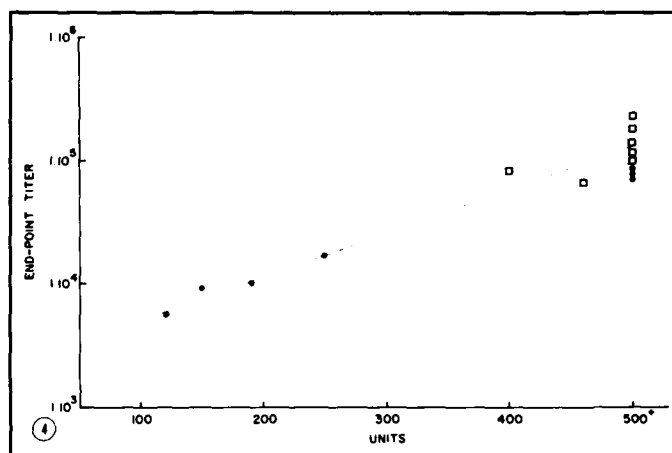


Fig 4—Correlation of JEV IgM ELISA end-point titers with JEV IgM ELISA units determined at a single screening dilution of 1:100.  $\square$  = early postviremia sera (3 to 10 days), and  $\bullet$  = later postviremia sera (11 to 14 days).

ance values were all  $< 0.3$ , and the absorbance was always less than when JEV antigen was used.

**Reproducibility of JEV IgM antibody bonding ELISA**—All 99 sera collected at the abattoirs were tested twice. The Pearson correlation coefficient of results between the 2 runs was 0.975. Using a cut-off value of 100 U, there was agreement between the 2 runs in 96 cases. Three borderline specimens that were positive in the 1st run, but negative in the 2nd run, contained 108/88, 120/85, and 150/93 U, for averages of 98, 102, and 121 U, respectively.

**Comparison of units and titers**—The relationship between units of JEV IgM activity (as determined by the color change at the single dilution of 1:100) and the end-point titer (as determined by serial dilutions and using a cut-off absorbance value of 0.2) was determined (Fig 4). Seven pairs of JEV IgM-positive sentinel pig sera were tested; the Pearson correlation coefficient was 0.93.

## Discussion

Immunoglobulin M production in mammals is transient; specific IgM appears quickly after the onset of in-

fection and then rapidly decreases.<sup>10</sup> Thus, detection of a high specific IgM in a serum specimen is indicative of an active or recent infection. In the pig, IgM is the least abundant of the 3 characterized Ig isotypes; serum concentrations of IgM in adult swine are typically 1 to 2 mg/ml.<sup>11</sup> The serum half-life of porcine IgM has been estimated to be 3 to 6 days.<sup>12,13</sup> Until recently, lack of a ready supply of antisera to porcine  $\mu$  chain has prevented the development of simple and rapid assays for diseases of swine, based on the detection of specific IgM antibody. We used mouse monoclonal antibody against porcine  $\mu$  chain to capture IgM from serum onto a solid-phase system.

This type of antibody-capture system has several convenient features: (i) Over a wide range of dilutions of serum, from undilute to 1:1,000, all the capture sites on the solid phase are saturated with IgM. Excess IgM is washed out of the system along with other serum proteins. When a single dilution of serum in the saturation range is tested, the final color product is a direct measure of the specific activity (antigen binding per mole of isotype). For any given serum specimen, the end-point titer and the specific activity are closely correlated, because porcine sera are fairly homogeneous with regard to their concentrations of total IgM. (ii) Competition between IgM and other isotypes is eliminated in the antibody-capture system because only IgM is bound; the other isotypes are washed out of the system along with excess IgM and other serum proteins. (iii) Crude antigens can be used in the antibody capture system, because only relevant antigens are bound onto the lawn of IgM molecules. Irrelevant impurities are washed out of the system.

One problem posed by highly sensitive assays for specific IgM is the definition of a cut-off value for recent infections. Although the peak of JEV IgM decreases rapidly after infection in human beings, low antibody activity often persists for years.<sup>4</sup> We arbitrarily selected a cut-off value for a recent JEV infection in pigs as equal to the mean specific activity of 5 porcine sera collected exactly 2 weeks after viremia and defined a weak-positive control serum with this specific activity as the cut-off standard of 100 U. It is improbable that many pigs maintain serum values of JEV IgM higher than this for a long period. Using this cut-off, none of 25 JEV HAI seronegative pigs and 7 (9%) of 74 JEV HAI-seropositive young pigs in abattoirs in Thailand had detectable serum JEV IgM antibodies.

Swine IgM synthesized in response to infection with

other flaviviruses may cross-react weakly with JEV and cause false-positive test results; this occurs in human beings.<sup>4</sup> False-positive test results can be identified by retesting borderline reactive specimens with equal antigen doses of JEV and other suspect flaviviruses. In our studies, all sera tested reacted more strongly with JEV antigen than with any of 4 other antigens prepared from viruses native to Thailand; all infections were probably caused by JEV.

The JEV IgM ELISA may be useful for the diagnosis of active or recent JEV infections in swine. Immunoglobulin M ELISA may also be useful for the diagnosis of other infections in swine.

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